

# Validity of immunohistochemistry method in predicting *HER-2* gene status and association of clinicopathological variables with it in invasive breast cancer patients

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Human epidermal growth factor receptor-2 is an important and prognostic factors and one of the most targeted proteins in breast cancer's therapy. There is no globally accepted method for determining its status. Here, we aimed to evaluate the immunohistochemistry method validity in predicting *HER-2* status by Fluorescence *in situ* hybridization method and investigate some clinicopathological variables association with *HER-2* amplification. A total of 190 *HER-2* 2+ and 3+ by immunohistochemistry (IHC) invasive breast cancer cases were enrolled in this study. Fluorescence *in situ* hybridization (FISH) was performed for these cases using FDA criteria and the association between clinicopathological variables and *HER-2* status evaluated. Study consisted of 190 invasive breast cancer patients (160 *HER-2* 2+ and 30 *HER-2* 3+). *HER-2* FISH amplification according to FDA criteria was found 27.5% (44/160 patients) in *HER-2* 2+ patients and 83.3% (25/30 patients) in *HER-2* 3+ patients. Tumors with *HER-2* amplification were more likely to be ER-negative (51.0% vs 31.2%,  $p = 0.013$ ) and PR-negative (52.9% vs 27.0%,  $p < 0.001$ ). This study showed that immunohistochemistry is not a good method for evaluating *HER-2* status and decision-making about trastuzumab therapy even with 3+ score patients. However, this result may not be too strong for IHC 3+ cases due to the limited number of these patients in this study.

Key words: Invasive breast cancer; human epidermal growth factor receptor-2; immunohistochemistry; fluorescent *in situ* hybridization; prediction.

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Breast cancer is responsible for 28% of the newly diagnosed cancer cases in women worldwide and second leading cause of cancer death in human (1, 2). Breast cancer with *HER-2* overexpression currently comprises 15–20% of all cases in the world (3). *HER-2/neu*, located on chromosome 17q21, encodes for the 185-kD transmembrane glycoprotein

*HER-2CA*, which is one of the most targeted proteins and plays a role in cell growth, differentiation, adhesion, motility, and signal conduction (4). *HER-2* amplification or overexpression has been demonstrated to be an independent parameter for bad prognosis. Even adjuvant hormonal therapy is not sufficient for these patients with positive estrogen receptors (5, 6).

*HER-2*-targeted therapies have significantly improved disease-free survival in women with

HER-2-positive cancers both in early and metastatic breast cancer (7, 8). Three HER-2-targeted agents, trastuzumab (Herceptin), lapatinib (Tykerb), and pertuzumab (Perjeta), have been made available in the past decade for the treatment of HER-2-positive metastatic breast cancer (9). The importance of precise detection of *HER-2* amplification is cleared with knowing the difficulties imposed by a 1-year course of treatment with Herceptin; this drug carries a substantial financial burden and perhaps, more importantly, introduces the risk of cardiotoxicity (10–12).

The US Food and Drug Administration (FDA) approved methodologies include the assessment of the protein level by immunohistochemistry (IHC) or gene copy count on the DNA level by *in situ* hybridization technology (ISH) (13, 14). Fluorescence *in situ* hybridization (FISH) is considered the gold standard for evaluating *HER-2* status, which has become essential in the era of personalized cancer therapy (13, 15). IHC is the most frequently used as initial laboratory test for HER-2 protein expression, because it is convenient and inexpensive. HER-2 IHC results are generally divided into four scale scores (range, 0–3+) on the basis of percentage of positive tumor cells and staining intensity. The US Food and Drug Administration (FDA) and American Society of Clinical Oncology/College of American Pathologists (ASCO/CAPs) recommends that HER-2 IHC scores of 0 and 1+ should be regarded as HER-2 negative and those with HER-2 3+ scores should be considered HER-2 positive. Tumors that show strong circumferential staining in <10% of tumor cells or incomplete and/or weak/moderate staining of >10% of the invasive tumor cells are categorized IHC 2+ score and are regarded as HER-2 equivocal and should be further assessed by FISH (16, 17).

The aims of the study were to evaluate the accuracy of immunohistochemistry as a method for predicting *HER-2* gene amplification status in a series of patients with IHC HER-2 2+, 3+ breast cancer and to study associations between *HER-2* status and clinicopathological factors.

## MATERIALS AND METHODS

### Patients

In this study we enrolled 190 patients diagnosed with invasive breast cancer including 160 HER-2 2+ and 30 HER-2 3+ in Alzahra hospital of Isfahan from March 2012 to August 2014. All patients were newly confirmed for invasive breast cancer status and have not received treatment. The patients and participants signed approved institutional review board consent forms before inclusion in the study. Patient clinical history and tumor characteristics were obtained from histopathology reports and

medical records. Gathered data included age histological grade, tumor size, regional lymph node status, lymphovascular invasion, estrogen receptor (ER), progesterone receptor (PR), and *HER-2* status. Nottingham criteria used to describe histological grade (18, 19). The tumor extent was measured by TNM staging according to the new staging system of the American Joint Committee on Cancer/International Union against Cancer (AJCC/UICC) (20). This study was approved by research and ethics committee of Isfahan University of medical sciences.

### Immunohistochemistry

Human epidermal growth factor receptor-2 IHC stains were performed with the HercepTest according to the manufacturer's instructions: the HercepTest (DAKO, Glostrup, Denmark). Each IHC stain was run with the kit control slides according to the manufacturers' instructions. Briefly, 4- $\mu$ m-thick mounted sections on slides from formaldehyde-fixed paraffin-embedded tissue blocks were deparaffinized by xylene solution for 5 min and rehydrated in graded alcohols for 3 min. After deparaffinization, tissue sections were placed in 0.1 M sodium citrate buffer (PH 6) for 40 min at 99 °C, after which the antigen was retrieved. The slides were then incubated with prediluted anti-*HER2* antibody for 1 h. After incubation, the sections were washed in PBS and incubated with horseradish peroxidase-conjugated secondary antibody. Color development was performed using 3,3'-diaminobenzidine, and the tissue samples were counterstained with hematoxylin. Negative and positive control slides were included in each assay. Samples were interpreted according to the ASCO/CAP guidelines: negative (0, 1+), weakly positive (2+), and strongly positive (3+). The results were interpreted by two independent pathologists.

All cases also underwent immunochemistry for hormone receptors. Primary antibodies used were mouse monoclonal anti-human ER antibody (1D5, Dako, Glostrup, Denmark) and mouse monoclonal anti-human progesterone receptor (PgR636, Glostrup, Denmark) antibody. A cutoff level of 10% or greater was defined as positive for ER and PR expression.

### FISH

The Cytocell HER-2 probe kit (LPS 001; Aquarius, Cytocell Technologies, Cambridge, UK) was used to analyze samples by FISH according to the supplier's instructions. First, the sections were baked overnight. Following deparaffinization by xylene, the slides were dehydrated and air-dried. The enzyme was freshly prepared by dilution. After protease digestion for 18 min, the slides and probes for HER-2 and the centromere of chromosome 17 (internal control) were treated at 72 °C for 2 min in denaturation solution simultaneously. The probes and target DNA were hybridized in a humidified chamber at 37 °C overnight. The slides were washed with Post-hybridization wash buffer by 0.4 $\times$  SSC at 72 °C for 2 min and once with 2 $\times$  SSC at room temperature for 30 seconds and counterstained with 4,6-diamidino-2-phenylindole. The number of chromosome 17 and HER-2 signals were scored for 20 tumor cell nuclei in the invasive tumor region which had been previously marked by the pathologist. *HER-2* gene status was evaluated based on the ratio

of HER-2 signals and chromosome 17 centromeric signals. A mean CEP17 signals per nucleus of  $\geq 3$  was considered as polysomy 17 (21). The FISH specimens were analyzed on a Nikon Eclipse 80i fluorescence microscope (Nikon Corp, Tokyo, Japan) with special filters. In our study, a case was regarded *HER-2* gene amplified if the ratio of *HER-2*/CEP17 was equal to or more than 2.0 as FDA recommendation.

### Statistical analysis

Pearson's chi-square test was performed to evaluate the association between clinicopathological variables and *HER-2* FISH positivity. Student's t-test was used to compare age between the *HER-2* negative and positive groups. Probability values  $<5\%$  were considered statistically significant.

For showing influence of risk factors on *HER-2* status (dependent variable) we used logistic regression model. In order to choose variables to involve in logistic regression, variables entered in logistic regression with *HER-2* status one by one and crude OR noted. After that variables with  $p$  value  $\leq 0.2$  considered for final multivariable logistic regression model.

All statistical analysis were performed using the PASW Gradpack 21 (SPSS Inc. Chicago, IL, USA).

## RESULTS

This study consisted of 190 invasive breast cancer patients (160 *HER-2* 2+ and 30 *HER-2* 3+). Study population median age was 48.63 years (range, 27–77 years). Histopathological grade was available in 135 patients (71.0%). 90 patients (47.3%) were grade 1 or 2 and 45 patients (25.2%) were grade 3. Hormone receptor (HR) status was available in all patients. ER was expressed in 141 patients (74.2%). PR also was expressed in 122 patients (64.2%). Hundred and twelve patients (58.9%) were ER and PR positive and 10 patients (5.2%) were ER negative and PR positive. Lymphovascular invasion (LVI) was seen in 45.7% (87 patients). The tumor size (T) and regional lymph node status (N) are shown in Table 1.

*HER-2* FISH amplification according to FDA criteria was found 27.5% (44/160 patients) in *HER-2* 2+ patients and 83.3% (25/30 patients) in *HER-2* 3+ patients. Totally 69 patients (36.3%) were *HER-2* FISH amplified (Table 1).

No significant differences existed between *HER-2* positive and *HER-2* negative groups with respect to

**Table 1.** Distribution of *HER-2* FISH results in IHC 2+ and 3+ breast cancer patients based on FDA guidelines

|        | FISH negative (%) | FISH positive (%) | Total |
|--------|-------------------|-------------------|-------|
| IHC 2+ | 116 (72.5)        | 44 (27.5)         | 160   |
| 3+     | 5 (16.7)          | 25 (83.3)         | 30    |
| Total  | 121 (63.7)        | 69 (36.3)         | 190   |

age (48.82 years vs 48.52 years,  $p = 0.854$ ), cancer location ( $p = 0.108$ ), lymphovascular invasion ( $p = 0.303$ ) or increased chromosome 17 copy number ( $p = 0.493$ ). Patients with *HER-2* FISH amplification tumors were not likely to have higher histological grades ( $p = 0.371$ ) compared with patients with unamplified tumors. Tumors with *HER-2* amplification were more likely to be ER-negative (51.0% vs 31.2%,  $p = 0.013$ ) and PR-negative (52.9% vs 27.0%,  $p < 0.001$ ) (Table 2).

A logistic regression model was used to reveal risk factors for *HER-2* amplification. The purpose of this logistic regression is to discover the impact of each clinicopathological variable on *HER-2* amplification singly; considering that other variables may affect the target variable's result. The association between clinicopathological variables and *HER-2* amplification is shown in Table 3. Tumor location, T stage, N stage, ER status, and PR status entered in final logistic regression model. Subjects with ER-positive expressions were less likely to exhibit *HER-2* amplification compared

**Table 2.** correlation of *HER-2* FISH results with clinicopathological variables in 190 breast cancer patients (significant  $P$  values are shown bold)

|                    | <i>HER-2</i><br>negative<br>n (%) | <i>HER-2</i><br>positive<br>n (%) | p                 |
|--------------------|-----------------------------------|-----------------------------------|-------------------|
| Patients           | 121 (63.7)                        | 69 (36.3)                         |                   |
| Age                | 48.82                             | 48.52                             | 0.854             |
| Location           |                                   |                                   |                   |
| Left               | 52 (57.8)                         | 38 (42.2)                         | 0.108             |
| Right              | 69 (69)                           | 31 (31)                           |                   |
| Histological grade |                                   |                                   |                   |
| 1–2                | 61 (67.8)                         | 29 (32.2)                         | 0.371             |
| 3                  | 27 (60)                           | 18 (40)                           |                   |
| Not evaluable      | 33 (60)                           | 22 (40)                           |                   |
| T stage            |                                   |                                   |                   |
| 1                  | 25 (71.4)                         | 10 (28.6)                         | 0.224             |
| 2                  | 86 (64.7)                         | 47 (35.3)                         |                   |
| 3                  | 8 (44.4)                          | 10 (55.6)                         |                   |
| 4                  | 2 (50)                            | 2 (50)                            |                   |
| N stage            |                                   |                                   |                   |
| 0                  | 53 (69.7)                         | 23 (30.3)                         | 0.329             |
| 1                  | 41 (64)                           | 23 (36)                           |                   |
| 2                  | 16 (51.6)                         | 15 (48.4)                         |                   |
| 3                  | 11 (57.9)                         | 8 (42.1)                          |                   |
| LVI                |                                   |                                   |                   |
| Negative           | 69 (67.0)                         | 34 (33.0)                         | 0.303             |
| Positive           | 52 (59.8)                         | 35 (40.2)                         |                   |
| ER status          |                                   |                                   |                   |
| Negative           | 24 (48.9)                         | 25 (51.1)                         | <b>0.013</b>      |
| Positive           | 97 (68.7)                         | 44 (31.3)                         |                   |
| PR status          |                                   |                                   |                   |
| Negative           | 32 (47.2)                         | 36 (52.9)                         | <b>&lt; 0.001</b> |
| Positive           | 89 (73.0)                         | 33 (27.0)                         |                   |
| Polysomy 17        |                                   |                                   |                   |
| Negative           | 98 (64.9)                         | 53 (35.1)                         | 0.493             |
| Positive           | 23 (59.0)                         | 16 (41.0)                         |                   |

**Table 3.** Logistic regression analysis of risk factors for *HER-2* FISH positive in 190 breast cancer patients (In all variables, first condition is supposed as reference) (significant P values are shown bold)

|                      |          | OR   | 95% CI    | p                |
|----------------------|----------|------|-----------|------------------|
| Age group            | Crude    | 1.12 | 0.62–2.02 | 0.711            |
| (<50, ≥50)           | Adjusted | –    | –         | –                |
| Location             | Crude    | 0.62 | 0.34–1.12 | <b>0.109</b>     |
| (Left, Right)        | Adjusted | 0.76 | 0.25–2.32 | 0.639            |
| Histological grade   | Crude    | 1.40 | 0.67–2.95 | 0.372            |
| (1–2, 3)             | Adjusted | –    | –         | –                |
| T stage              | Crude    | 1.58 | 0.95–2.62 | <b>0.072</b>     |
| (T1, T2–3–4)         | Adjusted | 1.63 | 0.96–2.77 | 0.067            |
| N stage              | Crude    | 1.28 | 0.94–1.72 | <b>0.107</b>     |
| (N0, N1–2–3)         | Adjusted | 0.64 | 0.32–1.28 | 0.215            |
| LVI                  | Crude    | 1.37 | 0.76–2.47 | 0.303            |
| (negative, positive) | Adjusted | –    | –         | –                |
| ER status            | Crude    | 0.44 | 0.22–0.85 | <b>0.014</b>     |
| (negative, positive) | Adjusted | 0.24 | 0.07–0.87 | <b>0.030</b>     |
| PR status            | Crude    | 0.33 | 0.18–0.61 | <b>&lt;0.001</b> |
| (negative, positive) | Adjusted | 0.32 | 0.14–0.74 | <b>0.008</b>     |

**Table 4.** *HER-2* gene amplification in IHC 2+ and 3+ breast cancer patients with Polysomy 17 (mean CEP17 signals per nucleus of ≥3)

|        | FISH negative (%) | FISH positive (%) | Total |
|--------|-------------------|-------------------|-------|
| IHC 2+ | 22 (62.9)         | 13 (37.1)         | 35    |
| 3+     | 1 (25)            | 3 (75)            | 4     |
| Total  | 23 (59.0)         | 16 (41.0)         | 39    |

with those with ER-negative expression (OR = 0.24; 95% CI = 0.07–0.87;  $p = 0.030$ ). The risk was also reduced in cases with PR-positive expressions than those with PR-negative expressions (OR = 0.32; 95% CI = 0.14–0.74;  $p = 0.008$ ).

There was polysomy 17 in 39 (20.5%) patients of which 35 (21.8%) and 4 (13.3%) cases were IHC 2+, and IHC 3+ respectively (Table 4).

## DISCUSSION

Human epidermal growth factor receptor-2, has been widely accepted as a prognostic and predictive marker in the management and treatment of breast cancer (22). Using trastuzumab supplement for neoadjuvant or adjuvant chemotherapy provides significant survival benefit in invasive breast cancer with *HER-2*-overexpressing tumor cells. However, for *HER-2*-negative cases, with any IHC score, trastuzumab offers no benefit and only contributes cardiotoxicity and waste of money (16, 17). The high cost and side effects of trastuzumab therapy demand that highly accurate, robust, sensitive, and cost-effective testing protocols be used in clinical settings (16, 23). The best method to evaluate *HER-2* status of breast cancer is still debated among pathologists. Because each technique has its own advantages and

disadvantages, a standard screening tool has not yet been determined. Several studies have proposed IHC as the first line screening method for *HER-2* status (15, 24). IHC staining is easy to perform and relatively inexpensive as a method for evaluating *HER-2* status. However, there is a wide range of inter- and intra-laboratory variation in its sensitivity and specificity (25, 26). Factors such as tissue fixation (and its impact on *HER-2* protein antigenicity), the scoring method, and the choice of antibody may contribute to a lower specificity and sensitivity of IHC (23, 27). Based on a recent study, false results could be decreased by standardization of these pre-analytic steps of IHC (28). Cases with weak positive staining (2+) by *HER-2* IHC represent a subgroup of patients that requires additional assessment with FISH. Applying updated ASCO/CAP recommendations would improve overall accuracy of *HER2* testing due to the widening of IHC 2+ cases (28). Some researchers have previously studied the probability of *HER-2* 2+ IHC breast cancers for *HER-2* amplification (17). In a study which was also in Iranian population 36% of *HER-2* 2+ samples were *HER-2* amplified (23). Another study with a relatively large population of 1735 breast cancer *HER-2* amplification were seen in 14% of patients (29). Other studies have also reported 30–35% *HER-2* amplification among IHC samples 2+ score (30, 31).

Numerous previous studies have reported that *HER-2* overexpression (IHC 3+) or *HER-2* amplification is associated with high tumor cell grade, absence of ER or PR expression, DNA aneuploidy, and high Ki-67 (32, 33). Many studies also investigated factors which could predict *HER-2* amplification in breast cancer patients. In a study which assessed correlation of different risk factors with *HER-2* amplification in IHC 2+ breast cancer patients, ER and PR positivity were shown to be related with negative *HER-2* status and better prognosis (17).

In our study, we integrated clinical and pathological factors from 190 breast cancer cases with IHC score of 2+ and 3+. All samples were routinely submitted for FISH analysis to determine the *HER-2* gene status. We found that 27.5% (44/160) of IHC 2+ cases were *HER-2* amplified. Which was a lower percentage in comparison with most of other studies. The reason is probably the fact that in the new ASCO/CAP *HER2* testing recommendation update used in this study, the IHC 2+ “equivocal” category has been expanded to include cases that would have previously been classified as 1+ negative or 0 negative (3). Positive correlation was also found between HR and *HER-2* status agreeing with previous studies. We expected to see higher tumor size (T stage) and regional lymph node involvement



(N stage) in *HER-2*-amplified patients, but they did not have significant association. However, T stage was very close to have a relation with *HER-2* positivity based on our results ( $p = 0.067$ ). Higher tumor grade and lymph node invasion also had no association with *HER-2* amplification. We also found five cases with IHC 3+ score to be *HER-2* negative on FISH. Based on established literature data it can be assumed that the way and duration of antigen retrieval and fixation can lead to inconsistent and incorrect IHC results (34). FISH assay is less prone to fixation and laboratory errors than IHC tests, making FISH assay more reproducible if signal interpretation is carried out by experienced pathologists. However, many studies stated that *HER-2* IHC false positive cases still benefit from adjuvant trastuzumab therapy (35).

Polysomy 17 is common among invasive breast cancer patients, however, the exact effect of polysomy 17 on *HER-2* amplification remains controversial (21). Some studies have reported that breast cancer patients with polysomy 17 respond to trastuzumab therapy independently of *HER-2* amplification (36), however, other researchers believe that polysomy 17 patients without *HER-2* amplification do not benefit from anti *HER-2* agents (13). Prevalence of polysomy 17 is reported in the range of 3–46% using the definition of  $\geq 3$  CEP per cell (37). In this study polysomy 17 was seen in 20.5% of patients. Also polysomy 17 is reported to be more common in IHC 2+ cases which agrees the findings of our study (38).

Some limitations were observed in this study. First we did not include Ki-67 and p-53 in breast cancer risk factors while many studies did show that they can predict *HER-2* status. The other limitation was few numbers of patients, especially IHC 3+ cases which was due to the FDA criteria to perform FISH for IHC 2+ patients only. Our IHC 3+ cases which FISH was performed for them were those who had a relative contraindication for Herceptin therapy like patients with high blood pressure or heart failure; Therefore these patients may not be representative of IHC +3 patients in general. In a recently meta-analysis, *HER-2* IHC 0/1+ and 3+ cannot be absolutely considered as negative and positive (39). In our study results also 16.7% (5/30) of patients with 3+ IHC score were not *HER-2* amplified which confirmed this fact.

In conclusion, IHC can not be the best method for evaluating *HER-2* amplification in breast cancer patients even in patients with 3+ score. Studying alternative methods for detecting *HER-2* amplification should be considered. Researches with larger groups and more predicting risk factors must perform in different populations.

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